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Biochemistry

A series of prostaglandin F_2-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalyzed mechanism

(eicosanoids/oxidative stress/lipid peroxidation/mass spectrometry)

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ABSTRACT **Increasing attention has focused on the role of free radicals derived from oxygen in the pathophysiology of a wide variety of disorders. One of the well-recognized targets of free radical-induced injury is peroxidation of lipids. Using a variety of approaches, we have found that a series of prostaglandin F_2-like compounds are produced *in vivo* in humans by a non-cyclooxygenase mechanism involving free radical-catalyzed peroxidation of arachidonic acid. Levels of these compounds in normal human plasma and urine range from 5 to 40 pg/ml and 500 to 4000 pg/mg of creatinine, respectively. In rats, their formation was found to increase as much as 200-fold in association with marked free radical-catalyzed lipid peroxidation induced by administration of CCl_4 and diquat. To explore whether these prostanoids can exert biological activity, the effects of one of the compounds formed by this mechanism, 8-epi-prostaglandin $F_{2\alpha}$, was examined in the kidney in the rat. Infusion of 8-epi-prostaglandin $F_{2\alpha}$ into a peripheral vein (5 μg/kg per min) or intrarenally (0.5–2.0 μg/kg per min) resulted in marked parallel reductions in renal blood flow and glomerular filtration rate. That the formation of these prostanoids is catalyzed by free radicals and that they can exert potent biological activity suggest that these prostanoids may participate as pathophysiological mediators in oxidant injury. Quantification of these compounds may also provide a noninvasive approach to assess oxidant status in humans. That the formation of these prostanoids occurs independent of the catalytic activity of the cyclooxygenase enzyme suggests that there may be limitations at times regarding the reliability of the use of cyclooxygenase inhibitors to assess the role of prostaglandins in certain pathophysiological processes.**

Considerable evidence indicates an important role of reactive free radicals derived primarily from oxygen in the pathophysiology of a wide spectrum of disorders including atherosclerosis, ischemia–reperfusion injury, inflammatory disease, cancer, and aging (1). One of the well-recognized targets of oxidative injury is peroxidation of lipids. Autoxidation of fatty acids under certain conditions *in vitro* results in the formation of prostaglandin-like compounds (2–4). Recently we found that arachidonoyl-containing lipids in plasma readily undergo free radical-catalyzed peroxidation *in vitro*, resulting in the formation of a series of prostaglandin F_2 (PGF_2)-like compounds (5). This appeared to be a very facile process. Therefore, we explored the possibility that these prostanoids may also be produced *in vivo* by a similar mechanism independent of the catalytic activity of the cyclooxygenase enzyme.

EXPERIMENTAL PROCEDURES

Analysis of PGF_2-Like Compounds in Plasma and Urine. PGF_2-like compounds were analyzed following TLC purification by GC/negative-ion chemical ionization and electron ionization MS (5). Catalytic hydrogenation and formation and analysis of cyclic boronate derivatives were carried out as described (5).

Assessment of the Effect of Cyclooxygenase Inhibition on the Endogenous Production of PGF_2-Like Compounds. Three human volunteers were employed in these studies. The following drugs were administered for 4 days: ibuprofen (1000 mg four times a day), naproxen (1000 mg four times a day), and indomethacin (200 mg four times a day). Among the three volunteers, ibuprofen was administered three times, naproxen once, and indomethacin twice. At least 4 plasma samples and 1–4 urine samples were obtained and analyzed at the end of each treatment period and compared to 21 plasma samples and 7 urine samples obtained during periods of no drug treatment.

Animal Models of Free Radical-Induced Lipid Peroxidation. Endogenous lipid peroxidation was induced by administration of diquat (19.5 μmol/kg) to Se-deficient rats and CCl_4 (2 ml/kg) to normal rats, as described (6, 7).

Biological Effects of 8-epi-$PGF_{2\alpha}$ in the Rat Kidney. 8-epi-$PGF_{2\alpha}$ (a generous gift from Gordon Bundy of Upjohn, Kalamazoo, MI) was infused into the jugular or left renal of euvolemic male Munich-Wistar rats prepared as described (8). Glomerular filtration rate and renal plasma flow were measured 30 min after the initiation of the infusion (8).

RESULTS

Analysis of PGF_2-Like Compounds in Human Plasma and Urine. Previously, analysis of fresh human plasma for F-type prostaglandins revealed a series of peaks presumably representing PGF_2-like compounds ranging from approximately 5 to 40 pg/ml (5). Analysis of fresh human urine also revealed a similar series of peaks ranging from approximately 500 to 4000 pg/mg of creatinine (Fig. 1). The pattern of these peaks in both fresh plasma and urine was very similar to the pattern of peaks previously documented to represent F-type prostanoids generated *in vitro* in plasma during storage (5).

Indirect evidence was obtained suggesting that these peaks in both fresh plasma and urine in fact represented PGF_2-like compounds. First, analysis of the compounds from both sources as a [$^2H_9$]trimethylsilyl ether derivative and following catalytic hydrogenation indicated that all of the compounds had three hydroxyl groups and two double bonds. In addition, analysis of an approximately equal mixture of the compounds from both urine and fresh plasma with the PGF_2-like compounds generated *in vitro* revealed perfect cochromatography on the capillary GC column, suggesting identity (data not shown).

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Abbreviations: PG, prostaglandin; BHT, butylated hydroxytoluene.
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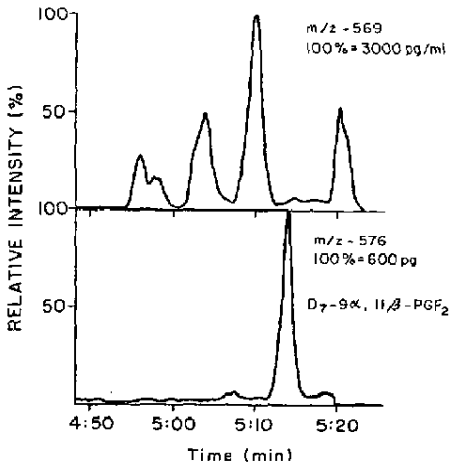

FIG. 1. Selected-ion current chromatogram obtained from GC/negative-ion chemical ionization MS analysis of PGF_2-type compounds in normal human urine. Compounds were analyzed as pentafluorobenzyl ester, trimethylsilyl ether derivatives with selected-ion monitoring of the M−181 (loss of the pentafluorobenzyl moiety) ions m/z 569 for endogenous PGF_2-type compounds and m/z 576 for the [$^2H_7$]9α,11β-PGF_2 internal standard (5). Levels of the endogenous compounds ranged from 700 to 3000 pg/ml. GC elution times are shown as min:sec. D, deuterium.

More definitive evidence for the identity of these compounds was then obtained from analysis of the compounds purified from 1 liter of normal human urine by electron ionization MS. This revealed multiple mass spectra of compounds eluted from the capillary GC column over an approximately 20- to 25-sec period characteristic of the electron ionization mass spectra of F-type prostaglandins (9). This period of 20–25 sec is the same approximate duration over which the m/z 569 peaks were eluted during selected-ion monitoring analysis (see Fig. 1). Important structural information was obtained from detailed analysis of these mass spectra and is discussed subsequently.

Evidence That These F-Type Prostanoids Are Produced *in Vivo*. The above data provided compelling evidence that the compounds detected in fresh plasma and urine are in fact PGF_2-like compounds. We then addressed whether these compounds are actually produced *in vivo* or whether they are formed *ex vivo* as was previously found to occur in plasma during storage (5). Butylated hydroxytoluene (BHT), 0.002%, inhibits the formation of these compounds *in vitro* by >90% (5). However, the presence of 0.002% BHT in the syringe into which blood was drawn did not suppress the levels of the compounds measured in fresh human plasma (n = 4). To address the unlikely possibility that the compounds were instantaneously formed in the needle of the syringe before the blood came into contact with BHT, we demonstrated that the levels of these compounds were essentially identical in fresh plasma that was analyzed immediately and after standing 2 hr at room temperature (n = 4). Similarly, the presence or absence of 0.002% BHT in the urine-collection containers did not influence the levels measured (n = 3). Furthermore, in contrast to what had been previously found to occur in plasma, levels of these compounds measured in urine did not increase during incubation of urine at 37°C for 7 days (n = 2) or during storage of urine for 6 months at −20°C (n = 3). With each of the above interventions, levels quantified in paired samples in each group varied by a mean of 10% or less.

Although the above findings provided evidence that these compounds are not formed *ex vivo*, the possibility that they may arise from dietary lipid intake was considered. To examine this, three normal volunteers were fed a diet consisting solely of glucose polymers and water for 4 days. However, levels of the compounds measured in urine collected at the end of the period of the glucose-polymer diet (891 ± 395 ng/mg creatinine) were essentially the same as the levels measured in urine from the same volunteers on an unrestricted diet (872 ± 217 ng/mg of creatinine).

Studies Related to the Mechanism Involved in the Formation of These Prostanoids *in Vivo*. To investigate the mechanism by which these prostanoids are produced *in vivo*, we first assessed whether the catalytic activity of cyclooxygenase is involved by determining whether their formation could be suppressed by treatment with cyclooxygenase inhibitors. Plasma levels during treatment (39.6 ± 11.2 pg/ml) were found to be essentially the same as levels measured in the absence of treatment (36.3 ± 12.9 pg/ml). Similarly, levels measured in urine in the absence of drug treatment (1991 ±

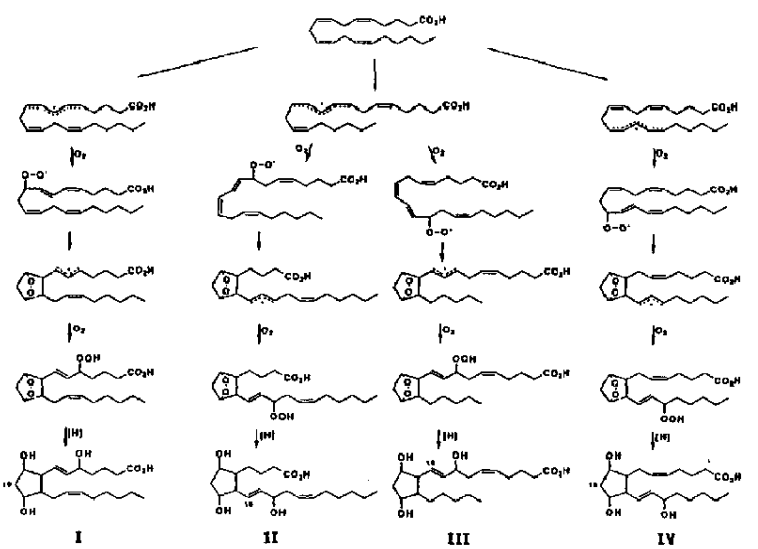

FIG. 2. Proposed mechanism for the non-cyclooxygenase formation of PGF_2-like compounds. For simplicity, stereochemistry is not specified. Four PGF_2-like regioisomers can be formed, each of which can comprise a mixture of eight racemic diastereomers. Reprinted with permission from ref. 5 (copyright Academic Press).

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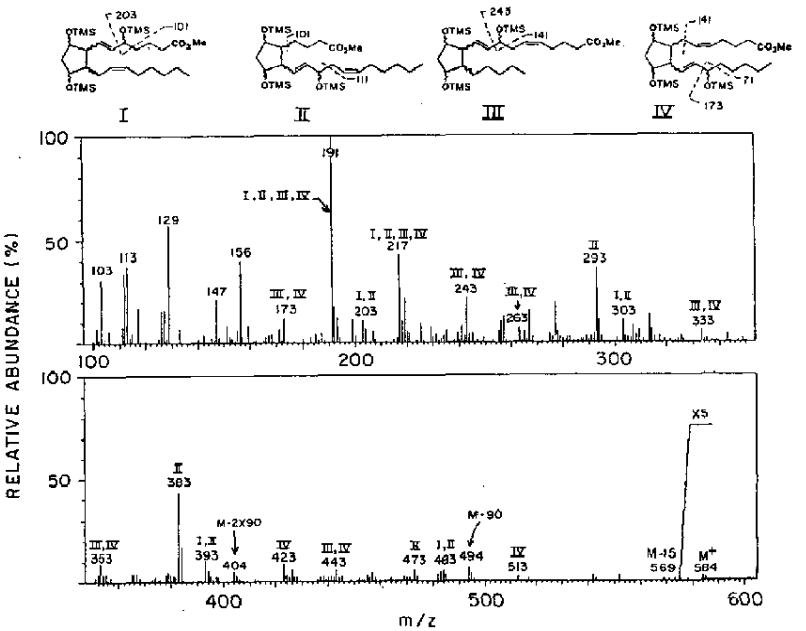

FIG. 3. Electron ionization mass spectrum obtained from analysis of human urine for PGF_2-like compounds as methyl ester, trimethylsilyl (TMS) ether derivatives. The origin of the particular ions present and their assignment to the individual PGF_2 regioisomers are indicated at the top of the figure and summarized in Table 1.

1523 pg/mg of creatinine) were not suppressed during treatment with cyclooxygenase inhibitors (2305 ± 1650 pg/mg of creatinine). In these studies, a high degree of cyclooxygenase inhibition *in vivo* was documented by demonstrating that each drug resulted in a 67–99% inhibition in the excretion of the major urinary metabolite of cyclooxygenase-derived PGD_2 (10). The PGD_2 metabolite was quantified by a highly accurate GC/MS assay (17).

That these F-type prostanoids in fresh plasma and urine comigrate on capillary GC with PGF_2-like compounds generated *in vitro* during storage of plasma suggests a common mechanism of formation. The mechanism that previously was envisioned to explain the formation of these compounds *in vitro* is shown in Fig. 2 (5). This involves the formation of bicyclic endoperoxide intermediates that are directly reduced to four PGF_2-like regioisomers, each of which can theoretically comprise eight racemic diastereomers. Detailed analysis of the electron ionization mass spectra obtained of the compounds in human urine mentioned previously provided evidence that the formation of these compounds *in vivo* also occurs by the same mechanism (Fig. 3). Prominent ions that would be predicted to be generated from the four regioisomers result from fragmentation α to the trimethylsilyl ether carbons and additional losses of Me_3SiOH (90 Da). The origins of these ions are shown in the structures at the top of Fig. 3 and are listed in Table 1. The assignment of these predicted ions to specific regioisomers I–IV is indicated above the ions in the mass spectrum. The *m/z* 191 ion (Me_3SiO^+=CH—$OSiMe_3$) is a rearrangement ion that is frequently a base ion of many PGF_2 compounds, and the *m/z* 217 ion (Me_3SiO—CH=CH=O^+SiMe_3) is usually a prominent ion in compounds with a PGF ring (9). Thus, these two ions can arise from all four regioisomers. In the mass spectrum shown, prominent ions that would be predicted to arise from each of the four regioisomers are present. Therefore, this mass spectrum was interpreted to be a mixed mass spectrum of all four of the regioisomers eluted simultaneously from the GC column.

The PGF_2-like compounds generated in plasma *in vitro* were found to predominantly have cis cyclopentane-ring hydroxyls in that they formed a cyclic boronate derivative (5). This indicated that they arose primarily from direct reduction of the endoperoxide intermediates in contrast to a mechanism involving isomerization of the endoperoxides to PGE- and PGD-like compounds followed by reduction to F-ring compounds, which would yield a mixture of compounds with cis and trans cyclopentane-ring hydroxyls. Using this same approach, it was found that the PGF_2-like compounds in fresh plasma and urine also predominantly have cis cyclopentane-ring hydroxyls (data not shown).

Endogenous Production of PGF_2-Like Prostanoids in Animal Models of Free Radical-Induced Lipid Peroxidation. To further substantiate the formation of these compounds *in vivo* by this mechanism, we assessed their production in two well-defined animal models of free radical-catalyzed lipid peroxidation. Diquat undergoes redox cycling *in vivo*, resulting in the production of free radicals, including the superoxide anion. Administration of diquat to Se-deficient rats leads to

Table 1. Proposed fragmentations for the formation of individual ions assigned to regioisomers I–IV in Fig. 3

Regioisomer I	Regioisomer II	Regioisomer III	Regioisomer IV
483 = M − 101	473 = M − 111	443 = M − 141	513 = M − 71
393 = M − 101 − 90	383 = M − 111 − 90	353 = M − 141 − 90	423 = M − 71 − 90
303 = M − 101 − (2 × 90)	293 = M − 111 − (2 × 90)	263 = M − 141 − (2 × 90)	333 = M − 71 − (2 × 90)
213 = M − 101 − (3 × 90)	203 = M − 111 − (3 × 90)	243	243 = M − 71 − (3 × 90)
203		173 = M − 141 − (3 × 90)	173
			(443, 353, 263: see regioisomer III)

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marked lipid peroxidation (6). Administration of CCl_4 to normal rats leads to the formation of $\cdot CCl_3$ radicals that catalyze peroxidation of lipids (7). Administration of both diquat and CCl_4 resulted in up to 200-fold increases in circulating levels of these compounds (Fig. 4). The pattern of m/z 569 peaks in the plasma from both diquat- and CCl_4-treated rats was essentially the same as the pattern shown in Fig. 1. Five rats were also treated with indomethacin prior to administration of CCl_4, with a dosage regimen previously shown to inhibit cyclooxygenase activity in rats by >90% (5 mg/kg at 24, 12, and 2 hr prior to administration of CCl_4; ref. 11). Indomethacin failed to suppress CCl_4-induced production of these prostanoids (Fig. 4*B*), again confirming that cyclooxygenase is not involved in their formation. The presence of BHT (0.002%) or superoxide dismutase (1500 units/ml of blood) in the blood-drawing syringes also did not affect plasma levels of the compounds measured, indicating that the increased levels measured in the plasma were not a result of formation *ex vivo*. Treatment of the rats with the reducing agent methylene blue (50 mg/kg, i.p.) prior to administration of CCl_4 significantly reduced plasma levels of PGF_2 compounds by a mean of 51% ($P < 0.0005$), further supporting a free radical mechanism of formation of these compounds.

Biological Effects of 8-Epi-$PGF_{2\alpha}$ in the Kidney. Although these compounds possess a cyclopentane prostane ring, like prostaglandins, there are unique differences in side-chain structures and stereochemistry of these compounds compared to cyclooxygenase-derived prostaglandins. Therefore, it was of interest to determine whether these compounds exert biological activity analogous to cyclooxygenase-derived prostaglandins. Autoxidation of fatty acids *in vitro* yields bicyclic endoperoxides in which the side chains are predominantly oriented cis (12). One such compound whose formation is favored by this mechanism, 8-epi-$PGF_{2\alpha}$ (12), has been chemically synthesized. Direct support for its formation *in vivo* was obtained by demonstrating that synthetic 8-epi-$PGF_{2\alpha}$ perfectly comigrates on capillary GC with the major PGF_2 peak eluted 4 sec earlier than [$^2H_7$]9α,11β-PGF_2 (see Fig. 1). Levels of these PGF_2-like compounds in urine are quite high, in the nanogram-per-milliliter range. Although it remains to be determined, the origin of these compounds in the urine, analogous to cyclooxygenase-derived prostaglandins, may derive at least in part from local formation in the kidney (13). For these reasons it was of interest to examine whether 8-epi-$PGF_{2\alpha}$ induced any biological alterations in the kidney. 8-Epi-$PGF_{2\alpha}$ was initially infused into a peripheral vein in five rats at a dose of 5 μg/kg per min. This resulted in a 40.5 ± 0.9% reduction in renal blood flow and a 45.3 ± 0.9% reduction in glomerular filtration rate. These effects were not accompanied by changes in systemic blood pressure, indicating a selective action of 8-epi-$PGF_{2\alpha}$ on renal vasculature at these doses. To explore the concentration–response relationship of these effects, the concentration of 8-epi-$PGF_{2\alpha}$ was measured in plasma obtained from the abdominal aorta 30 min after initiation of one of these infusions and was found to be 34 nM.

The effect of intrarenal infusion of 8-epi-$PGF_{2\alpha}$ was then examined. This also caused a marked parallel reduction in both glomerular filtration rate and renal plasma flow in a dose-dependent fashion (Fig. 5). At the highest dose, 2 μg/kg per min, glomerular filtration rate and renal plasma flow fell to zero within 2 min after initiation of the infusion, urine flow ceased, and the kidney took on a pale and mottled appearance, indicative of severe vasoconstriction. At similar doses (2 μg/kg per min), intrarenal infusions of cyclooxygenase-derived $PGF_{2\alpha}$ and the biologically active metabolite of PGD_2, 9α,11β-PGF_2 (14), had no significant effect on renal blood flow or glomerular filtration rate (K.F.B., unpublished data).

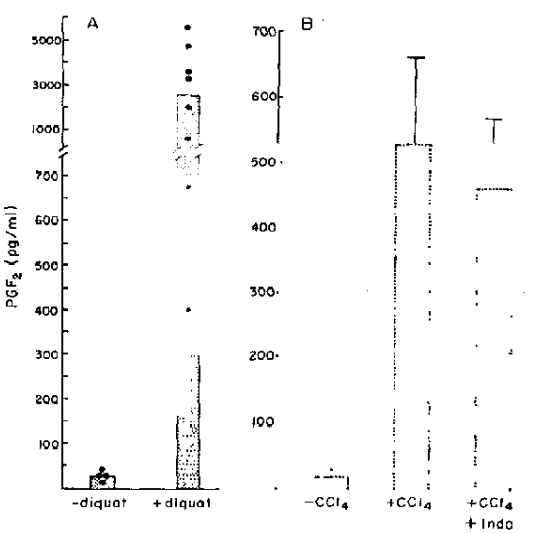

FIG. 4. (*A*) Levels of PGF_2-like prostanoids in plasma obtained from Se-deficient rats 90 min after administration of diquat compared with levels in Se-deficient rats not given diquat. Dots represent plasma levels in individual animals and the bars represent mean values. (*B*) Levels of PGF_2-like prostanoids in plasma obtained from normal rats 90 min after administration of CCl_4 with or without indomethacin (Indo) pretreatment compared with plasma levels measured in untreated rats. Results are expressed as mean ± SD (n = 5 for each group).

DISCUSSION

Although the catalytic activity of cyclooxygenase has been assumed to be obligatory for the endogenous production of prostaglandins, these studies have elucidated that there is an alternative, non-cyclooxygenase pathway that also leads to the formation of prostanoids *in vivo*, by a mechanism involving free radical-catalyzed peroxidation of arachidonic acid. This involves the formation of bicyclic endoperoxide intermediates that are then directly reduced to yield a series of F-type prostanoids. Although these studies focused on the identification of PGF_2-like compounds that are produced by this mechanism, the possibility exists that E-type and D-type prostanoids may also be formed as a result of isomerization of the endoperoxide intermediates. We have obtained preliminary evidence that this does occur *in vitro* in plasma, but whether this also occurs *in vivo* remains to be explored.

It is of interest that the levels of these non-cyclooxygenase-derived prostanoids in normal human plasma and urine are 1–2 orders of magnitude higher than those of cyclooxygenase-

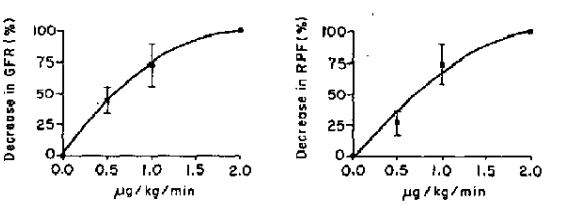

FIG. 5. Percent reduction in glomerular filtration rates (GFR) and renal plasma flow (RPF) during intrarenal infusions of 8-epi-$PGF_{2\alpha}$ in rats. Data are expressed as the mean (n = 2) for the 0 and 2 μg/kg per min doses and the mean ± SD (n = 4) for the 0.5 and 1.0 μg/kg per min doses.

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derived prostaglandins, indicating that this is not a minor pathway. An unexplained finding published by Nugteren in 1975 (15) may have relevance to this discovery. Using a novel mass spectrometric assay for the simultaneous determination of tetranor metabolites of all prostaglandins irrespective of the prostane-ring subtype from which they were derived, Nugteren (15) found an ≈10-fold higher level of total endogenous prostaglandin production in humans than could be accounted for from previous studies of prostaglandin metabolism. The explanation for this finding has always remained an enigma. In retrospect, this discrepancy may now be explained by the likely possibility that Nugteren's assay also detected tetranor metabolites of these non-cyclooxygenase-derived prostaglandin-like compounds.

The potential importance of this discovery encompasses three general areas. First, it is well recognized that there are limitations with many of the approaches currently available to assess oxidative stress *in vivo* (16). These PGF_2-like compounds are easily detectable in human biological fluids and their formation increases markedly in animal models of lipid peroxidation. Thus, quantification of these compounds may provide a useful noninvasive approach to assess oxidant status in humans.

Many of the underlying mechanisms responsible for the biological sequelae to oxidative stress are incompletely understood. In this regard, another potentially important aspect surrounding this discovery relates to the possibility that these prostanoids may participate as mediators in the pathophysiology of oxidative stress. In support of this possibility, we found that one of the compounds that can be formed by this mechanism, 8-epi-$PGF_{2\alpha}$, was biologically active. It was found to be an extremely potent renal vasoconstrictor in the low nanomolar range. It is approximately an order of magnitude more potent than leukotriene D_4, which is the most potent renal vasoconstricting eicosanoid known (8). In light of these findings, studies investigating additional biological actions of this prostaglandin and the bioactivity of the other PGF_2-like compounds formed by this mechanism will be of considerable interest.

Finally, there are potentially important pharmacological ramifications associated with these findings. The use of cyclooxygenase inhibitors is generally accepted as a valid approach to assess the role of prostaglandins in pathophysiological processes. However, in view of the finding that prostanoids are also formed endogenously by a cyclooxygenase-independent mechanism, it seems important to recognize that there may be limitations at times regarding the interpretation and reliability of this commonly used pharmacological intervention.

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